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(54) Title: NON-TOXIC IMMUNE STIMULATING ENTEROTOXIN COMPOSITIONS

(57) Abstract

Pyrogenic toxins, such as staphylococcal enterotoxins, modified in the disulfide loop region are provided. The modified toxins retain useful biological properties but have substantially reduced toxicity compared to the corresponding unmodified native toxin. The native pyrogenic toxins are typically modified by deletions within the disulfide loop region to produce modified enterotoxins having 100-fold or greater decrease in toxicity.

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NON—TOXIC IMMUNE STIMULATING
ENTEROTOXIN COMPOISITIONS

GOVERNMENT SUPPORT

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Health under NIH grant R11A128401. The United States Government has certain
rights in the invention.

BACKGROUND OF THE INVENTION

10   A group of biological agents termed superantigens has been described
based on their ability to stimulate monocytes and unprimed $CD4^+$ (MHC Class II
restricted) and $CD8^+$ (MHC Class I restricted) T—cells. Included with the
designation of superantigens are the enterotoxins of *Staphylococcus aureus*.

The enterotoxins of *Staphylococcus aureus* form a group of
15   serologically distinct proteins, originally designated A, B, C_1, C_2, C_3, D, E, G, and H.
Subsequently, a number of variants have been described. These proteins and toxic
shock syndrome proteins were originally recognized as the causative agents of
staphylococcal food poisoning. Ingestion of preformed enterotoxin in contaminated
food leads to the rapid development (within two to six hours) of symptoms of
20   vomiting and diarrhea that are characteristic of staphylococcal food poisoning.
Toxic shock syndrome toxin—1, TSST—1, a distantly related protein also produced
by *S. aureus*, is classically responsible for the toxic shock syndrome, although other
staphylococcal enterotoxins may result in the syndrome due to the induction of
cytokines.

25   Other agents which have been identified as superantigens include for
example, the staphylococcal exfoliative toxins, A and B; the mammary tumor virus
superantigen; rabies virus nucleocapsid protein; pyrogenic exotoxins A, B, C from *S.
pyrogens*; and the *Mycoplasma arthritides* mitogen. Additional biological agents
which have been demonstrated to have superantigen properties include the human
30   immunodeficiency virus (HIV), gp120 and peptides from HIV, mouse mammary
tumor virus and feline immunodeficiency virus. A Leishmania peptide antigen has
also been disclosed as a superantigen.

Superantigens, unlike conventional antigens, do not require
processing *in—vivo*. In general, superantigens have two binding regions, one of
35   which interacts with the Class II major histocompatibility complex (MHC) on the

antigen presenting cell and the other which interacts with the Vβ variable region of the T−cell receptor on CD4 and/or CD8 cells. Various enterotoxins bind to one or more of the different Vβ receptor epitopes. In contrast to conventional antigens, superantigens do not occupy the T−cells receptor cleft but are felt to bind to an

5 external region thus explaining the ability to activate a broad population of T−cells.

Enterotoxins produced by *Staphylococcus aureus* include a group of related proteins of about 20 to 30 Kd. The complete amino acid composition of a number of staphylococcal enterotoxins and streptococcal pyrogenic exotoxin has been reported (see e.g., PCT Patent Appl. No. WO 93/24136.)

10 Staphylococcal enterotoxins ("SEs") were initially classified on the basis of their antigenic properties into groups A, B, C1, C2, C3, D, and E. Subsequent relatedness was based on peptide and DNA sequence data. Among the staphylococcal enterotoxins, groups B and C are closely related and groups A, D, and E are closely related in amino acid sequence. SEC1, SEC2, and SEC3 and

15 related isolates share approximately 95% sequence similarity. Table 1 shows the alignment of the predicted sequences of the eight known SEC variants following cleavage of the signal peptide. The N−terminus of each of the mature proteins was verified by amino acid sequencing. Amino acid positions that contain residues that are not conserved among all SEC are indicated by asterisks. SEB and SEC are

20 approximately 45−50% homologous. In contrast, non−enterotoxin superantigens, TSST−1 and *Streptococcal Pyrogenic Enterotoxin C* (SPEC) share only approximately 20% primary sequence homology to SEC. Despite these differences, the tertiary structure of the various enterotoxins show nearly identical folds.

The staphylococcal enterotoxins A, B, C_1, C_2, C_3, D, E, G and H

25 share a common structural feature of a disulfide bond not present in other enterotoxins. Table 2 shows the position of the disulfide bond in a number of enterotoxins. Data in reference to the active sites of the enterotoxin molecule in relationship to biological activity, MHC binding, and TCR binding has been obtained. Sequence data demonstrate a high degree of similarity in four regions of

30 the enterotoxins (See Table 3). The peptides implicated in potential receptor binding correspond to regions 1 and 3 which form a groove in the molecule. Amino acid residues within and adjacent to the α_3 cavity of SEC3 have been shown to relate to T−cell activation.

Table 2.

LOCATION OF DISULFIDE LOOP IN STAPHYLOCOCCUS ENTEROTOXINS

ENTEROTOXIN	AMINO ACID RESIDUES	AMINO ACID SEQUENCE OF DISULFIDE LOOP
SEA	96–106	96→CAGGTPNKTAC
SEB	93–114	93→CYFSKKTNDINSHQTPKRKTC
SEC1	93–110	93→CYFSSKDNVGKVTGGKTC
SEC2	93–110	93→CYFSSKDNVGKVTGGKTC
SEC3 FRI 913	93–110	93→CYFSSKDNVGKVTGGKTC
SEC3 FRI 909	93–110	93→CYFSSKDNVGKVTSGKTC
SEC 4446	93–110	93→CYFSSKDNVGKVTGGKTC
SEC–Bovine	93–110	93→CYFSSKDNVGKVTGGKTC
SEC–Ovine	93–110	93→CCFSSKDNVGKVTGGKTC

The staphylococcal enterotoxins are potent activators of T–cells, resulting in proliferation and the generation of cytotoxic T–cells. SEA is a potent T–cell mitogen eliciting strong polyclonal activation at concentrations of 10^{-13} to 10^{-10} molar in human systems.

The staphylococcal enterotoxins, aside from the acute gastroenteritis and toxic shock syndrome associated with them, have been shown to have a variety of other beneficial biological effects. The biological effects of these agents and the toxic shock syndrome are due in part to the ability of staphylococcal enterotoxins to induce cytokines. Various cytokines described include IL–1, IL–2, and tumor necrosis factor ("TNF"). More recently SEB and toxic shock syndrome toxin ("TSST–1") have been shown to induce interleukin–12, an inducer of cell mediated immunity, in human peripheral blood mononuclear cells. (See Leung et al., *J Exp Med*, 181:747 (1995)). The antitumor activity of treating cancer in rabbits utilizing 40 to 60 µg/kg of a staphylococcal enterotoxin has been disclosed in PCT Patent Appl. Nos. WO 91/10680 and WO 93/24136.

Exposure to enterotoxin either *in–vitro* or *in–vivo* leads to depletion of T–cells having the appropriate Vβ receptor through programmed cell death in some strains of mice, specifically Balb/c and CBA/2. Cell death can be prevented by

high doses of retinol or RU−38486. Programmed cell death has not been observed upon exposure of human cells to enterotoxins.

Although the systemic lethal toxicity of enterotoxins has been related to their ability to induce cytokines, particularly IL−1, IL−2 and gamma interferon,

5   lethal toxicity also appears to be related to a synergistic activity with endogenous endotoxins and the ability of the liver to detoxify endotoxins. Although a number of animals have been utilized to evaluate lethality, the accepted model is the continous infusion over a period of time, usually 4 days, in rabbits. The direct toxic dose varies among various species. The 50% lethal dose of TSST−1 is approximately 50

10 µg/kg for Balb/c mice. Piglets, although showing clinical manifestations of toxic shock syndrome, tolerate doses of 100 µg/kg of TSST−1. TSST−ovine is known to be non−toxic at doses of 200 µg in rabbits.

In Dutch belted rabbits, intramuscular injection of 50 mg/kg of staphylococcal enterotoxin B caused death. Intravenous injection at 0.5 to 1.0 mg/kg

15 of enterotoxin A or B in rhesus macaques results in hypotension and death (Liu C.T., et al *Amer J Vet Res* 39:279 and 1213, 1978).

In contrast to other species, man is extremely sensitive to enterotoxins. One (1) mg of TSST−1, approximately 15 nanogram/kg, can be lethal for man. Therefore, the recommended doses currently proposed in the art for

20 treating man are unacceptable. There is a need, therefore, for mutant staphylococcal enterotoxins which are non−toxic at anticipated doses for man while still retaining desirable biological activity.

Several studies of staphylococcal enterotoxin have identified a number of biologically active modified or mutant enterotoxins with reduced toxicity.

25 Carboxymethylation of SEB results in a loss of gastrointestinal toxicity but not mitogenic activity. Studies with the TSST−1 have demonstrated the active site to be between amino acids residue 115 and 141. Point mutation of site 135 from histidine to alanine results in a loss of mitogenic activity and toxicity (See Bonventre P.F., et al. *Infect Immun* 63:509 (1995)). Studies with the staphylococcal enterotoxin SEC1

30 demonstrated that the disulfide bond between residue 93 and 110 is not required for activity (See Hovde et al., *Mol Microbiol* 13:897 (1994)). Studies of the molecular binding region of staphylococcal enterotoxin B using overlapping peptides demonstrated peptide 124 to 154 inhibited SEB induced mitogenic activity.

Based on the known biological activities of the toxic native

35 enterotoxins, it is desirable to create mutants which are at least 1000−fold or more

less toxic compared to native enterotoxins and retain biological activity. Recent studies have demonstrated that mutant enterotoxins can be produced which retain certain biological activities and which may be significantly less lethal as determined in rabbits. A mutant of the TSST−1 enterotoxin which differs in amino acid 136 and

5   is non−lethal at ten times the lethal dose of the native toxin (in rabbits), but retains biological activity has been disclosed. A number of mutants of SEC1, unable to form a disulfide bond, have been reported to be ten times less toxic than the native toxin while retaining biological activity. (See e.g., Hovde et al., *Molec Microbiol* 13:897 (1994)).

10

SUMMARY OF THE INVENTION

The present invention relates to modified versions of disulfide loop−containing bacterial pyrogenic toxins. The modified pyrogenic toxins retain useful biological properties but have substantially reduced toxicity (e.g., toxicity reduced

15   by at least about 10−fold) compared to the corresponding unmodified native toxin. Selected deletions within the disulfide loop region can produce modified toxins having a 100−fold or greater decrease in toxicity. The toxicity of the modified toxin can be measured based on a variety of parameters, including emetic response inducing activity, fever inducing activity, and lethality (as measured by LD_{50} in

20   Dutch Belted rabbits).

Examples of the present modified toxins include disulfide loop region deletion mutants of native toxins derived from *Staphylococcus aureus* or *Streptococcus pyrogenes*. Suitable native disulfide loop−containing toxins which may be modified according to the present invention include Type A, B, C, D and E

25   staphylococcal enterotoxins as well as streptococcal pyrogenic enterotoxin A ("SPEA") and streptococcal superantigen ("SSA") produced by *S. pyrogenes*.

The pyrogenic toxins constitute a family of exotoxins produced by species of gram positive cocci, such as Staphylococcus and Streptococcus. The pyrogenic toxins are characterized by shared ability to induce fever, enhance host

30   susceptibility to endotoxin shock, and induce T cell proliferation through action as superantigens. Examples of pyrogenic toxins include TSST−1, staphylococcal enterotoxins (SEs), and streptococcal pyrogenic exotoxins (SPEs). In addition to the activities listed above, some pyrogenic toxins have additional activities that are not shared by all pyrogenic toxins. For example, the staphylococcal enterotoxins induce

35   emesis and diarrhea when ingested. Structurally, the pyrogenic toxins have varying

degrees of relatedness at the amino acid and nucleotide sequence levels. A number of the pyrogenic toxins include a disulfide loop as a structural feature. The staphylococcal enterotoxins have a disulfide loop, as do some others in this family. Examples of other pyrogenic toxins that have a disulfide loop are the streptococcal

5   superantigen ("SSA") and streptococcal pyrogenic exotoxin A ("SPEA").

The pyrogenic toxins have varying degrees of relatedness which provides the basis for separating some of them informally into subgroups. One subgroup includes staphylococcal type B and C enterotoxins ("SEB" and "SEC"), as well as SPEA and SSA. These toxins share between about 49% to greater than 95%

10   amino acid sequence homology (Reda et al, Infect. Immun., 62:1867–1874: (1994)). Another subgroup of related pyrogenic toxins include staphylococcal type A and E enterotoxins (SEA and SEE) which are 83% homologous to each other (Couch et al, J. Bacteriol., 70:2954–2060 (19___), less so but significantly to SED (Bayles et al., J. Bacteriol., 171:4799–4806 (1989)). The amino acid sequences of this second

15   subgroup is more distantly related to SEB, SEC, SPEA, and SSA. Examples of pyrogenic toxins having disulfide bonds are present in both of these two subgroups. TSST–1 and streptococcal pyrogenic exotoxins B and C (SPEB and SPEC) are examples of a third subgroup of less related toxins. Although toxins from this third subgroup may share some conserved regions (see table 3) with toxins from the other

20   subgroups, there is little overall sequence homology between toxins in the third subgroup and the pyrogenic toxins in the other two subgroups. Neither TSST–1, SPEB nor SPEC includes a disulfide loop.

The disulfide loop region of a native pyrogenic toxin, such as a native staphylococcal enterotoxin, is generally modified through deletion of a number of

25   amino acid residues within the loop. The modification typically includes deletion of amino acid residues within the disulfide loop region and may include one or more substitutions and/or additions to the remaining loop residues. After modification, the disulfide loop region typically contains no more than about 10 and, preferably, no more than about 6 amino acids residues. In another embodiment of the invention,

30   a modified pyrogenic toxin is formed from a native pyrogenic toxin modified by deletion of at least 40% of the amino acid residues within the disulfide loop region, e.g., by deletion of 8 or more amino acid residues from the disulfide loop region of a native type C staphylococcal enterotoxin.

The present invention is also directed to isolated nucleic acids which

35   include a nucleotide sequence encoding a modified pyrogenic toxin.

DETAILED DESCRIPTION OF THE INVENTION

The current invention relates to modified versions of pyrogenic toxins, such as staphylococcal enterotoxins produced by modifications which include deletions in the disulfide bond region. The present invention describes the

5   feasibility of obtaining mutant pyrogenic toxins which retain biological activity but demonstrate significantly lower toxicity at doses well in excess of the normal lethal dose and levels anticipated for human therapeutic use.

The modified pyrogenic toxins are derived from a native pyrogenic toxin having a disulfide loop. The terms "disulfide loop" and "disulfide loop

10   region" are used interchangeably herein. As employed in this application, these terms refer to the sequence of about 10 to about 30 amino acid residues forming a loop defined by a disulfide bond in a native pyrogenic toxin. The term "disulfide loop region" also refers to the corresponding portion of the sequence of a modified pyrogenic toxin which has been produced by deletion, substitution or addition of one

15   or more amino acid residues of the disulfide loop of a native pyrogenic toxin. The disulfide loop region is defined to begin with the N-terminal Cys residue and end with the C-terminal Cys residue of the loop, e.g., amino acid residues 93-110 of staphylococcal enterotoxin C1. As used herein, the positions of the disulfide loop region for a given native pyrogenic toxin are numbered beginning with the N-

20   terminal cyteine residue in the loop, e.g., position 93 of type B or C staphylococcal enterotoxins is also referred to herein as position 1 of the disulfide loop region.

The modification of the disulfide loop typically includes deletion of at least about 40% of the amino acid residues within the disulfide loop. For example, this generally results in the deletion of at least about 8 amino acid residues

25   from the disulfide loop region of an SEC. Examples of native staphylococcal enterotoxin which can be modified to form the present low toxicity toxins include type A, B, C, D, E, G, and H staphylococcal enterotoxins. Type C staphylococcal enterotoxins such as staphylococcal enterotoxin C1, staphylococcal enterotoxin C2, staphylococcal enterotoxin C2, staphylococcal enterotoxin C-MNCopeland,

30   staphylococcal enterotoxin C-4446, staphylococcal enterotoxin C-bovine (GenBank Accession No. L13374), staphylococcal enterotoxin C-canine (GenBank Accession No. V19526) and staphylococcal enterotoxin C-ovine (GenBank Accession No. L13379) are particularly suitable enterotoxins for modification by deletion of a portion of the disulfide loop region to form a staphylococcal enterotoxin with

35   decreased toxicity.

The modified disulfide loop region generally contains no more than about 10 amino acid residues and, preferably no more than 6 amino acid residues. For example, a type C staphylococcal enterotoxin, such as staphylococcal enterotoxin C1, can be modified to delete amino acid residues 98−106 (residues 6−

5   14 of the disulfide loop) to form a modified staphylococcal enterotoxin having substantially reduced toxicity. An even greater reduction in toxicity is produced by deleting amino acid residues 95−106 (disulfide loop residues 3−14) of staphylococcal enterotoxin C1. Both of these mutants, despite having substantially reduced toxicity, are biologically active as evidenced by their ability to stimulate the

10   uptake of thymidine by human peripheral blood mononuclear cells.

In addition to deletion of a number of the disulfide loop residues, substitution of a cysteine residue for the residue at position 2 of disulfide loop can contribute to a decrease in the toxicity of a pyrogenic toxin such as a type C staphylococcal enterotoxin. In a preferred embodiment, staphylococcal enterotoxin

15   C1 can be modified so that in addition to deletion of a substantial portion of the residues in the loop region, the amino acid residue at position 2 of the loop, Tyr−94, is replaced by a cysteine.

Other examples of preferred embodiments of the invention include modified staphylococcal enterotoxins having no more than 10 amino acid residues

20   and, preferably, no more than 6 amino acid residues in the disulfide loop region. Particularly preferred examples include staphylococcal enterotoxins with no more than about 9 amino acid residues which include the sequence Cys−Gly−Lys−Thr. One example of such a mutant is staphylococcal enterotoxin C1 modified to have the disulfide loop region sequence Cys−Cys−Gly−Lys−Thr−Cys.

25   The methods of this invention employed in preparing mutant enterotoxins, and their screening, analysis, and purification are known in the art and described herein. Site directed mutagenesis can be carried out by initially cloning the SEC1 structural gene SEC_{MNDON} on a 1.4 Kb MindITI−BamH1 restriction fragment. A unique $SphI$ restriction site (5'−GCATGC−3') can be introduced into

30   SEC_{MNDON} in the region coding for the disulfide loop at nucleotides 301−306 (5'− GTAGGT−3') using a commercially available kit (Altered Sites in $vitro$ Mutagenesis System, Promega). Potential mutants may be screened by $SphI$ digestion and confirmed by nucleic acid sequence analysis.

Select SEC_{MNDON} deletion mutants were cloned into an $E.$ $coli$ cell

35   line using pMIN164 an $E.$ $coli$ − $S.$ $aureus$ shuttle vector. To facilitate protein

purification, recombinant plasmids from *E. coli* RR1 transformants were transformed into *S. aureus* RN4220 by standard protoplast transformation techniques. Plasmids containing cloned toxin genes were maintained in *S. aureus* RN4220 under erythromycin (50 mg/ml) selection. For purification of native and

5   mutant derivatives of the toxin recombinants, *S. aureus* RN4220 cultures were grown in dialyzable beef heart media supplemented with 1% glucose buffer (330 mM glucose; 475 mM $NaHCO_3$; 680 mM NaCl; 137 mM $Na_2HPO_4.H_2O$; 28 mM L‑glutamine) and erythromycin (50 mg/ml) followed by ethanol precipitation.

Purification of ethanol precipitated proteins was accomplished by

10   preparative flat bed isoelectric focusing (IEF). Following the IEF run, proteins in select fractions were pooled, dialyzed to remove the amphylotes and then visually assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS‑PAGE).

The toxicity and biological activity of the mutants was evaluated

15   using standard methods known to those skilled in the art as described below. The emetic activity of SEC1, and the SEC1 mutant toxins was determined by a modification of the standard monkey feeding assay using young adult pigtail monkeys (*M. nemestrina*). The animals were manually restrained while the toxin was administered through a nasogastric tube (Infant feeding tube; Becton Dickinson,

20   Rutherford, NJ). Toxins were screened for retention of emetic activity at a dose of 10 µg/Kg which is approximately 100 times the minimal emetic dose for SEC1. Non‑emetic toxins were tested for residual emetic activity at a high dose of 250 µg/Kg.

The mitogenic capacities of mutant toxins were compared to that of

25   SEC1 native toxin by using human peripheral blood mononuclear cells (PMBC) in a standard 4‑day assay. Solutions of native and mutant toxin were added in triplicate to PBMC cell suspensions, 1×10^6 cells/ml, in 96‑well tissue culture plates. This mixture of cells and toxin was then incubated at 37°C under atmospheric conditions of 6% CO_2 for 72 hours. [^{3}H]‑thymidine (New Research Products, Boston, MA) at

30   a concentration of 1 µCi/25 µl in a complete RPMI medium was added to each well and allowed to incubate under the same conditions for an additional 18‑24 hours. After incubation, radiolabeled cellular nucleic acids were harvested onto glass fiber filters (Skatron, Sterling, VA) using a semi‑automatic cell harvester (Skatron). Lymphocyte proliferation was quantitated by measuring incorporation of [^{3}H]‑

thymidine into cellular DNA using a liquid scintillation counter (TRI–CARB 1500 Liquid Scintillation Center, Packard, Rockville, MD).

The ability of a staphylococcal enterotoxin to induce a fever response and enhance susceptibility of lethal endotoxic shock can be determined *in vivo* using a standard rabbit model (Bohach et al., Infect. Immun., 55, 428 (1987)). Following conditioning in a test rack and having baseline body temperature recorded, adult New Zealand White rabbits can be initially intravenously injected with a native or mutant toxin at a concentration of 10 µg/kg in sterile physiological saline. Sterile saline and purified SEC1 toxin are typically used as negative and positive controls respectively. Following toxin injection, rabbit body temperature is generally monitored rectally every hour for four hours. Four hours after initial treatment, an intravenous injection of lipopolysaccharide ("LPS") from *Salmonella typhimurium* (Difco Laboratories, Detroit, Michigan) is administered intravenously at a concentration of 10 µg/Kg in sterile saline. Animals are then observed for signs of shock and mortality for 48 hours after LPS injection.

Cytokine induction may be determined by utilizing isolated mononuclear cells from heparinized venous blood. Briefly, heparinized venous blood is obtained and layered onto lymphocyte separation medium. The tubes are spun and the mononuclear layer is harvested, washed in PBS, resuspended in RPMI containing 10% FCS and adjusted to 1×10^6 cells per ml. Aliquots of 100 µl are typically placed into 96–well microtiter plates. The enterotoxins were added in 100 µl to a final concentration of 1 ng/ml. Following incubation at 37°C for 48–72 hours, the supernatant was harvested can be assayed for cytokines using commercially available kits from R&D Systems Minneapolis, MN.

The invention will be further described by reference to the following examples. These examples illustrate but do not limit the scope of the invention that has been set forth herein. Variation within the concepts of the invention will be apparent.

EXAMPLE 1

The SEC1 structural gene, *SEC$_{MNDON}$*, was previously cloned on a 1.4 Kb *HindIII–BamH1* restriction fragment (Bohach et al., Infect. Immun., 55, 428 (1987)). A unique *SphI* restriction site (5'–GCATGC–3') was introduced into *SEC$_{MNDON}$* in the region coding for the disulfide loop at nucleotides 301–306 (5'–GTAGGT–3') using a commercially available kit (Altered Sites *in vitro* Mutagenesis

System, Promega). Potential mutants were screened by *SphI* digestion and
confirmed by nucleic acid sequence analysis.

EXAMPLE 2

5   The unique *SphI* site was used to linearize the SEC_{MNDON} gene so that
bi-directional deletions could be generated using *Bal-31* exonuclease (Boehringer
Mannheim, Indianapolis, IN). *Bal-31* generated deletion mutant plasmids were
ligated and transformed into *E. coli* TG1. Transformants growing on LB-AMP (125
µg/ml) with in-frame stable deletions were detected by screening with SEC1
10   specific rabbit antisera in immunodiffusion and analyzed by nucleic acid sequence
analysis. Sequencing reactions were done using *Sequenase Version 2.0,* a
commercially available kit (U.S. Biochemical Corp., Cleveland, Ohio).

      Three deletion mutants were chosen for detailed analysis based on the
size and location of the deletions in the loop region of the toxin; SEC1-4AA,
15   SCE1-9AA, and SEC1-12AA. Following purification, each of the disulfide loop
mutant toxins could be distinguished from wild type SEC1 on the basis of size when
analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

      The nucleic acid and amino acid sequences for SEC1 and three
deletion mutants are shown in Table 4.
20

EXAMPLE 3

Mutant Toxin Purification

      Select SEC_{MNDON} deletion mutants were cloned into *E. coli* RR1
using pMIN164, an *E. coli* – *S. aureus* shuttle vector. To facilitate protein
25   purification, recombinant plasmids from *E. coli* RR1 transformants were
transformed into *S. aureus* RN4220 by standard protoplast transformation
techniques. Plasmids containing cloned toxin genes were maintained in *S. aureus*
RN4220 under erythromycin (50 µg/ml) selection. For purification of the
recombinantly produced native and mutant derivatives of the toxin, *S. aureus*
30   RN4220 cultures were grown in dialyzable beef heart media supplemented with 1%
glucose buffer (330 mM glucose; 475 mM $NaHCO_3$; 680 mM NaCl; 137 mM
$Na_2HPO_4.H_2O$; 28 mM L-glutamine) and erythromycin (50 µg/ml) followed by
ethanol precipitation.

      Purification of ethanol precipitated proteins was accomplished by
35   preparative flat bed isoelectric focusing ("IEF"). Following the IEF run, proteins in

select fractions were pooled, dialyzed to remove the ampholytes and then visually assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

5   **EXAMPLE 4**

Emesis Assay

Assays of the emetic activity of SEC1 and the modified SEC1 toxins were conducted using a modification of the standard monkey feeding assay using young adult pigtail monkeys (*M. nemestrina*). The animals were manually restrained

10   while the toxin was administered through a nasogastric tube (Infant feeding tube; Becton Dickinson, Rutherford, NJ). Toxins were screened for retention of emetic activity at a dose of 10 μg/Kg which is approximately 100 times the minimal emetic dose for SEC1. Non–emetic toxins were tested for residual emetic activity at a high dose of 250 mg/Kg.

15   It has been previously shown that the minimal emetic dose of wild type SEC1 for *M. nemestrina* was 0.1 μg/Kg. For initial experiments in which the emetic ability was tested, loop mutant toxins were administered at 10 μg/Kg. This insured an excess of toxin over the wild type SEC1 minimal emetic dose. Following intragastric toxin inhibition, animals were observed for at least 12 hours for an

20   emesis response. SEC1–12AA did not show emesis at the 10 μg/Kg concentration and was subsequently tested for emesis at a higher toxin concentration, 250 μg/Kg. Even at this higher doseage level, the SEC1–12AA loop mutant toxin showed no emetic response (See Table 5).

Table 5.

Emetic Response[c] of SEC1 Loop Deletion Mutants

Dose[a]	SEC1	SEC1-4aa	SEC1-9aa	SEC1-12aa
250 µg/Kg	ND[b]	ND	ND	0/2
10 µg/Kg	2/2	2/2	2/2	0/2
1 µg/Kg	2/2	2/2	0/1	ND

5   [a] µg Toxin/Kg Body Weight
     [b] ND = Not Determined
     [c] Number animals exhibiting emetic response/Total number of animals

EXAMPLE 5

10   **Pyrogenicity and Enhancement of Lethal Endotoxic Shock**

         The SEC1 mutant toxin's ability to induce a fever response and
enhance susceptibility of lethal endotoxic shock was determined *in vivo* using a
previously described rabbit model. Following 1 hour of conditioning in a test rack
and having baseline body temperature recorded, adult New Zealand White rabbits
15   were initially intravenously injected with SEC1 or SEC1 mutant toxin at a
concentration of 10 µg/Kg in sterile physiological saline. Sterile saline and purified
SEC1 toxin were used as negative and positive controls, respectively. Following
toxin injection, rabbit body temperature was monitored rectally every hour for four
hours. Four hours after initial treatment an intravenous injection of
20   lipopolysaccharide (LPS) from *Salmonella typhimurium* (Difco Laboratories,
Detroit, Michigan) was administered intravenously at a concentration of 10 µg/Kg in
sterile saline. Animals were watched for signs of shock and mortality for 48 hours
after LPS injection.

         Wild type SEC1, administered at both 10 µg/Kg and 1 µg/Kg, showed
25   a typical temperature rise (Table 6) as well as an enhanced susceptibility to
endotoxic shock (Table 7).

Table 6.

Maximum Temperature Rise in °C in Rabbits Given Noted Amount of Native SEC1, Compound to SEC1−4, SEC1−9, or SEC1−12 Mutant

	SEC1	SEC1−4aa	SEC1−9aa	SEC1−12aa
100 µg	ND	ND	ND	0.6
10 µg	1.6	1.46	0.9	0.45
1 µg	1.3	1	0.43	ND
0.1 µg	1.05	0.65	ND	ND
0.01 µg	0.475	0.425	ND	ND

Table 7.

Lethality in Dutch Belted Rabbits of Native SEC1 and SEC1 Deletion Mutants

Dose (µg)	SEC1	SEC1−4	SEC1−9	SEC1−12
100	ND	ND	ND	0/3
10	3/3	3/3	2/3	0/3
1	2/2	3/3	0/3	ND
0.1	3/3	2/3	ND	ND
0.01	1/4	0/3	ND	ND

SEC1−12AA loop mutant toxin concentration was increased to 100 µg/Kg after test animals showed both reduced pyrogenic effects and no susceptibility to endotoxin shock at the initial dose tested, 10 µg/Kg. Following this log fold increase of the SEC−12AA mutant toxin to 100 µg/Kg it was seen that the mutant toxin induced a slight temperature increase over the initial SEC1−12AA loop mutant toxin concentration tested but was not lethal in the assay.

EXAMPLE 6

Mitogenicity

        The mitogenic capacity of mutant toxin was compared to that of
SEC1 native toxin by using human peripheral blood mononuclear cells (PMBC) in a
5   standard 4–day assay (Bohach et al., Infect. Immun., 55, 428 (1987)). Human
peripheral blood mononuclear cells were isolated from 30 to 60 ml of heparinized
blood obtained by venipuncture. The blood was mixed 1:1 with Dulbecco's
Phosphate Buffered Saline (PBS) (without Calcium or magnesium) and layered onto
Fico/Lite (density 1.079 g/ml, Atlanta Biologicals, Norcross, GA) and centrifuged at
10   400 x g for 20 minutes. The interface containing the mononuclear cells was
removed and washed 3 times with PBS. After the last wash, the pellet was
resuspended in Hank's Balanced Salt solution without calcium or magnesium and
layered on Fetal Bovine Serum (Atlanta Biologicals, Norcross, GA) and centrifuged
at 100 x g for 10 minutes to remove the platelets. The cells were washed in Hank's
15   Balanced Salt solution, Cat. No. M1211–021–LV (Atasca, IL). A cell count was
done by Trypan Blue exclusion.

        Solutions of native and mutant toxins were added in triplicate to
PMBC cell suspensions, 1×10^6 cells/ml, in 96 well tissue culture plates. This
mixture of cells and toxin was then incubated at 37°C under atmospheric conditions
20   at 6% CO_2 for 72 hours. [^{3}H]–thymidine (New Research Products, Boston, MA) at
a concentration of 1 µCi/25 µl in a complete RPMI medium was added to each well
and allowed to incubate under the same conditions for an additional 18–24 hours.
After incubation, radiolabeled cells were harvested onto glass fiber filters (Skatron,
Sterling, VA) using a semi–automatic cell harvester (Skatron). Lymphocyte
25   proliferation was quantitated by measuring incorporation of [^{3}H]–thymidine into
cellular DNA using a liquid scintillation counter (TI–CARB 1500 Liquid
Scintillation Counter, Packard, Rockville, MD).

        Using SEC1 wild type toxin as a control it was determined that the
SEC1–12AA loop mutant toxin showed mitogenic activity at a reduced effectiveness
30   compared to native SEC1 and the SEC1–4 and SEC1–9 mutants in stimulating
PMBC cells (see Table 8).

Table 8.

**SEC1 Loop Mutant Mitogeneicity [³H]−Thymidine Uptake
(in CPM) of Enterotoxin Stimulated Human Peripheral
Blood Mononuclear Cels**

Dose	SEC1	SEC1−4AA	SEC1−9AA	SEC1−12AA
0.1 pg	1918	2067	1067	978
1 pg	8533	7453	2360	954
0.01 ng	14138	15495	7682	1200
0.1 ng	21557	23689	21481	6500
1 ng	30329	28892	28951	9400
0.01 μg	33089	31640	23333	2700

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains and are hereby incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

Table 1.
Amino Acid Sequence of Selected Staphylococcal Enterotoxins

```
                  40
                   *
SEC1            1 ESQPDPTPDELHKASKFTGLMENMKVLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEGLAKKYKDE  80
SEC2            1 ESQPDPTPDELHKSSEFTGTMGNMKYLYDDHYVSATKVMSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE  80
SEC3-FRI913    1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE  80
SEC3-FRI909    1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNINDKKLNNYDKVKTELLNEDLANKYKDE  80
SEC-MNCopeland 1 ESQPDPTPDELHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE  80
SEC-4446       1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKRLKNYDKVKTELLNEDLAKKYKDE  80
SEC-bovine     1 ESQPDPTPDELHKASKFTGLMENMKVLYDDRYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE  80
SEC-ovine      1 ESQPDPTPDELHKASKFTGLMENMKVLYDDRYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE  80

                  120
SEC1           81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC2           81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC3-FRI913    81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC3-FRI909    81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC-MNCopeland 81 VVDVYGSNYYVNCYFSSKDNVGKVTSGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC-4446       81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC-bovine     81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC-ovine      81 VVDVYGSNYYVNCCFFSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160

                  200
SEC1          161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
SEC2          161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
SEC3-FRI913   161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
SEC3-FRI909   161 IKARNFLINKKNLYEFNSSPYETGYIKFIESNGNTFWYDMMPAPGDKFDQSKYLMIYKDNKMVDSKSVKIEVHLTTKNGX 240
SEC-MNCopeland161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX 240
SEC-4446      161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX 240
SEC-bovine    161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX 240
SEC-ovine     161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX 240
```

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Table 3A.

Conserved Regions of Enterotoxin Molecules*

Toxin	Residue #	Region 1		Toxin	Residue #	Region 3
SEA	79	K Y K G K K V D L Y G		SEA	147	K K N V T V Q E L D L Q A R R Y L
SEB	76	K Y K D K Y V D V F G		SEB	152	K K K V T A Q E L D Y L T R H Y L
SEC1	76	K Y K D E V V D V Y G		SEC1	151	K K S V T A Q E L D I K A R N F L
SEC2	76	K Y K D E V V D V Y G		SEC2	151	K K S V T A Q E L D I K A R N F L
SEC3	76	K Y K D E V V D V Y G		SEC3	151	K K S V T A Q E L D I K A R N F L
SED	74	H F K S K N V D V Y P		SED	142	K K N V T V Q E L D A Q A R R Y L
SEE	76	K Y K G K K V D L Y G		SEE	144	K K E V T V Q E L D L Q A R H Y L
SPEA	70	L F K D K N V D I Y G		SPEA	137	K K M V T A Q E L D Y K V R K Y L
SPEC	63	F K R D D H V D V F G		SPEC	124	K D I V T F Q E I D F K I R K Y L
TSST-1	56	F T K G E K V D L N T		TSST-1	121	K K - - - Q - L - I
				TSST-1	129	L D F E I R H Q L

* From Hoffmann et al., *Infect Immunol* 62:3396 (1994).

Table 3B.

Conserved Regions of Enterotoxin Molecules (Cont.)*

Toxin	Residue #	Region 2		Toxin	Residue #	Region 4
SEA	106	C M Y G G V I L H D N N		SEA	209	L L R I Y R D N K T I N S E
SEB	113	C M Y G G V T E H N G N		SEB	213	Y L M M Y N D N K M V D S K
SEC1	110	C M Y G G I T K H E G N		SEC1	213	Y L M M Y N D N K T V D S K
SEC2	110	C M Y G G I T K H E G N		SEC2	213	Y L M M Y N D N K T V D S K
SEC3	110	C M Y G G I T K H E G N		SEC3	213	Y L M I Y K D N K M V D S K
SED	101	C T Y G G V T P H E G N		SED	204	Q L R I Y S D N K T L S T E
SEE	103	C M Y G G V T L H D N N		SEE	206	L L R I Y R D N K T I N S E
SPEA	98	C I Y G G V T N H E G N		SPEA	197	Y L M I Y K D M E T L D S N
SPEC	85	Y I Y G G I T P A Q N N		SPEC	184	I F A K Y K D N R I I N M K
TSST-1	83	F Q I S G V T N T E K L		TSST-1	179	P P I N I D E I K T I E A E

* From Hoffmann et al., *Infect Immunol* 62:3396 (1994).

TABLE 4.

SEC1 LOOP MUTANTS

AMINO ACID #	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110
SEC1 (wild type) AMINO ACID NUCLEIC ACID	Cys TGC	Tyr TAT	Phe TTT	Ser TCA	Ser TCC	Lys AAA	Asp GAT	Asn AAT	Val GTA	Gly GGT	Lys AAA	Val GTT	Thr ACA	Gly GGT	Gly GGC	Lys AAA	Thr ACT	Cys TGT
SEC1 Loop Deletion Mutants							301	–	306									
-4 A.A. MUTANT	Cys TGC	Tyr TAT	Phe TTT	Ser TCA	Ser TCC	Lys AAA	Asp GAT	Asn AAT	Ala GCA	———				Gly GGT	Gly GGC	Lys AAA	Thr ACT	Cys TGT
-9 A.A. MUTANT	Cys TGC	Tyr TAT	Phe TTT	Ser TCA	Ser TCC	———									Gly GGC	Lys AAA	Thr ACT	Cys TGT
-12 A.A MUTANT	Cys TGC	Cys T--	———											GT	Gly GGC	Lys AAA	Thr ACT	Cys TGT

WHAT IS CLAIMED IS:

1.   A modified pyrogenic toxin derived from a native disulfide loop–containing pyrogenic toxin, wherein the modified toxin comprises a disulfide loop region containing no more than 10 amino acid residues.

2.   The modified toxin of claim 1 wherein the native disulfide loop–containing pyrogenic toxin is a staphylococcal toxin or a streptococcal toxin.

3.   The modified toxin of claim 2 wherein the staphylococcal toxin is a type A, B, C, D, E, G, or H staphylococcal enterotoxin.

5.   The modified toxin of claim 2 wherein the streptococcal toxin is streptococcal pyrogenic exotoxin A or streptococcal superantigen.

6.   The modified toxin of claim 1 wherein the disulfide loop region contains no more than 6 amino acid residues.

7.   The modified toxin of claim 1 wherein the native disulfide loop–containing pyrogenic toxin is a type C staphylococcal enterotoxin.

8.   The modified toxin of claim 7 wherein the modification comprises a deletion of at least 8 amino acid residues within the disulfide loop region.

9.   The modified toxin of claim 8 wherein the modification comprises a deletion of amino acid residues 98–106.

10.   The modified toxin of claim 7 wherein the modification comprises deletion of at least 12 amino acid residues within the disulfide loop region.

11.   The modified toxin of claim 10 wherein the modification comprises deletion of amino acid residues 95–106.

12.   The modified toxin of claim 7 wherein the type C staphylococcal enterotoxin is staphylococcal enterotoxin C1.

13. The modified toxin of claim 12 comprising a cysteine residue at position 94.

14. The modified toxin of claim 7 wherein the staphylococcal enterotoxin is staphylococcal enterotoxin C1, staphylococcal enterotoxin C2, staphylococcal enterotoxin C2, staphylococcal enterotoxin C-MNCopeland, staphylococcal enterotoxin C-4446, staphylococcal enterotoxin C-bovine, staphylococcal enterotoxin C-canine or staphylococcal enterotoxin C-ovine.

15. The modified toxin of claim 1 comprising a cysteine residue at position 2 of the disulfide loop region.

16. The modified toxin of claim 1 having substantially decreased toxicity in comparison to the native toxin.

17. The modified toxin of claim 16 having an emetic response inducing activity decreased by at least about 100-fold in comparison to the native toxin.

18. The modified toxin of claim 16 having an fever inducing activity decreased by at least about 100-fold in comparison to the native toxin.

19. The modified toxin of claim 16 having an LD_{50} in Dutch Belted rabbits which is at least about 100-fold higher than the native toxin.

20. A modified pyrogenic toxin derived from a native disulfide loop-containing pyrogenic toxin, wherein the modification comprises deletion of at least about 40% of the amino acid residues within the disulfide loop of the native toxin.

21. The modified pyrogenic toxin of claim 20 comprising a cysteine residue at position 2 of the disulfide loop region.

22. The modified pyrogenic toxin of claim 20 wherein the native pyrogenic toxin is a type C staphylococcal enterotoxin.

23. An isolated nucleotide acid comprising a nucleotide sequence encoding a modified pyrogenic toxin derived from a native disulfide loop-containing pyrogenic

toxin, wherein the modified toxin comprises a disulfide loop region which includes no more than 10 amino acid residues.

24.   An isolated nucleotide acid comprising a nucleotide sequence encoding a modified staphylococcal enterotoxin derived from a native staphylococcal enterotoxin, wherein the modification comprises deletion of at least about 40% of the amino acid residues within the disulfide loop region of the native enterotoxin.

25.   A modified pyrogenic toxin derived from a native type C staphylococcal enterotoxin, wherein the modified toxin comprises a disulfide loop region which includes no more than 10 amino acid residues.

26.   The modified toxin of claim 25 wherein the disulfide loop region is Cys–Cys–Gly–Lys–Thr–Cys.

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(57) Abstract

Pyrogenic toxins, such as staphylococcal enterotoxins, modified in the disulfide loop region are provided. The modified toxins retain useful biological properties but have substantially reduced toxicity compared to the corresponding unmodified native toxin. The native pyrogenic toxins are typically modified by deletions within the disulfide loop region to produce modified enterotoxins having 100–fold or greater decrease in toxicity.

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A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00; C07H 21/04; C07K 1/00, 2/00
US CL :530/300, 350; 536/23.7

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Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350; 536/23.7

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CA, EMBASE
terms: pyrogenic, entertoxin, modified, fragment, staphylococcus, streptococcus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97/36932 A1 (PHARMACIA & UPJOHN AB) 09 OCTOBER 1997 (09/10/97), see entire document.	1-26
Y	NISHI et al. B Cell Epitope Mapping of the Bacterial Superantigen Staphylococcal Enterotoxin B. Journal of Immunology. 01 January 1997, Vol. 158, No. 1, pages 247-254.	1-26
Y	ALAKHOV et al. Identification of functionally active fragments of staphylococcal enterotoxin B. Eur. J. Biochem. 01 November 1992, Vol. 209, pages 823-828.	1-26

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BINEK et al. Localisation of the mitogenic epitope of staphylococcal enterotoxin B. J. Med. Microbiol. March 1992, Vol. 36, pages 156-163.	1-26